Reversibility of Ultrastructural Changes of Cultured Human Nasal Epithelial Cells Induced by Tumor Necrosis Factor-α

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ABSTRACT

Tumor necrosis factor (TNF)-α can induce ultrastructural changes in cultured human nasal epithelial cells. The aims of this study are to determine the recovery time from the changes induced by TNF-α and observe whether various degrees of changes will result in a complete return to normal status. The recovery of cilia was observed on the third day after cessation of adding TNF-α and the normalization of secretory cell area was observed on the fifth day. The morphologic changes induced by TNF-α were reversible except with the concentration of 100 ng/ml. The results suggest that the ultrastructural changes induced by TNF-α are mostly reversible and that this reversibility corresponds with the *in vivo* state.

KEY WORDS : Ultrastructure · Reversibility · TNF-α.

INTRODUCTION

Tumor necrosis factor (TNF)-α plays a critical role in a normal host's resistance to infections and to the growth of malignant tumor. It serves not only as immunostimulants but also as a mediator of inflammatory response.[1] The induction of cytokines and involved factors in the inflammatory reaction is the most important one of the many actions of TNF-α.[2]

Recently, the role of this pro-inflammatory cytokine has been studied intensively. Cyclo-oxygenase 2 and nitric oxide are increased in human pulmonary epithelium by the action of TNF-α.[3] In the human nasal mucosa, interleukine 1 and 8 are increased and T, B and NK cells are activated by the action of TNF-α.[4][5]

Morphologic studies show that TNF-α can induce the morphologic changes in endothelial cells of bovine aorta[6] and human renal epithelial cells.[7] In a previous study, the authors reported the ultrastructural changes of cultured human nasal epithelial cells (HNEC) induced by TNF-α.[8] The ultrastructural changes by TNF-α in HNEC were observed from 1 ng/ml through 100 ng/ml. Damage of cilia, an increase of mitochondria and either intercellular space or intercellular vacuoles were noted in transmission electron microscopy. Observing the surface area of cells, the area of secretory cells were seen to increased with 1 ng/ml through 100 ng/ml in scanning electron microscopy. The above patterns of various changes, such as the damage of cilia, an increase of mitochondria and either intercellular space or intercellular vacuoles were similar to the *in vivo* state.

In human, the morphologic changes of the epithelial cells by inflammation is reversible in most cases.[9] The changes by TNF-α in cultured HNEC are similar to those of *in vivo* state, but the degree of changes vary according to the concentration of TNF-α. Concerning the reversibility of the changes, we wondered whether the morphologic changes of the epithelial cells induced by TNF-α are completely normalized despite the various degree of the changes and also evaluated the duration of recovery time from the changes induced by TNF-α. The purpose of this study was to observe the reversibility of the ultrastructural changes of HNEC induced by TNF-α with variances in the time sequence and concentration.

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MATERIALS AND METHODS

Culture of HNEC

Human nasal inferior turbinates and polyps obtained during the turbinectomy and polypectomy were used as culture materials. The HNEC culture was prepared for use of the floating method.[10][11] Firstly, cells were isolated using 0.1% pronase

solution (Sigma Chemical Co., St. Louis, MO, USA) for 16 - 24 hours. The isolated cells contained epithelial cells, stromal cells, endothelial cells and others. To isolate only the epithelial cells, the mixed cells were incubated in a suspended solution in a plastic petri-dish at 37 ℃ for one hour. The epithelial cells obtained were plated in a 35 mm diameter dish coated with type I collagen gel (Collaborative Research Inc., Bedford, MA, USA). The media was changed at three or four day intervals. After nine or ten days, the confluent cells on the collagen gel were transferred to 60 mm dish and floated within

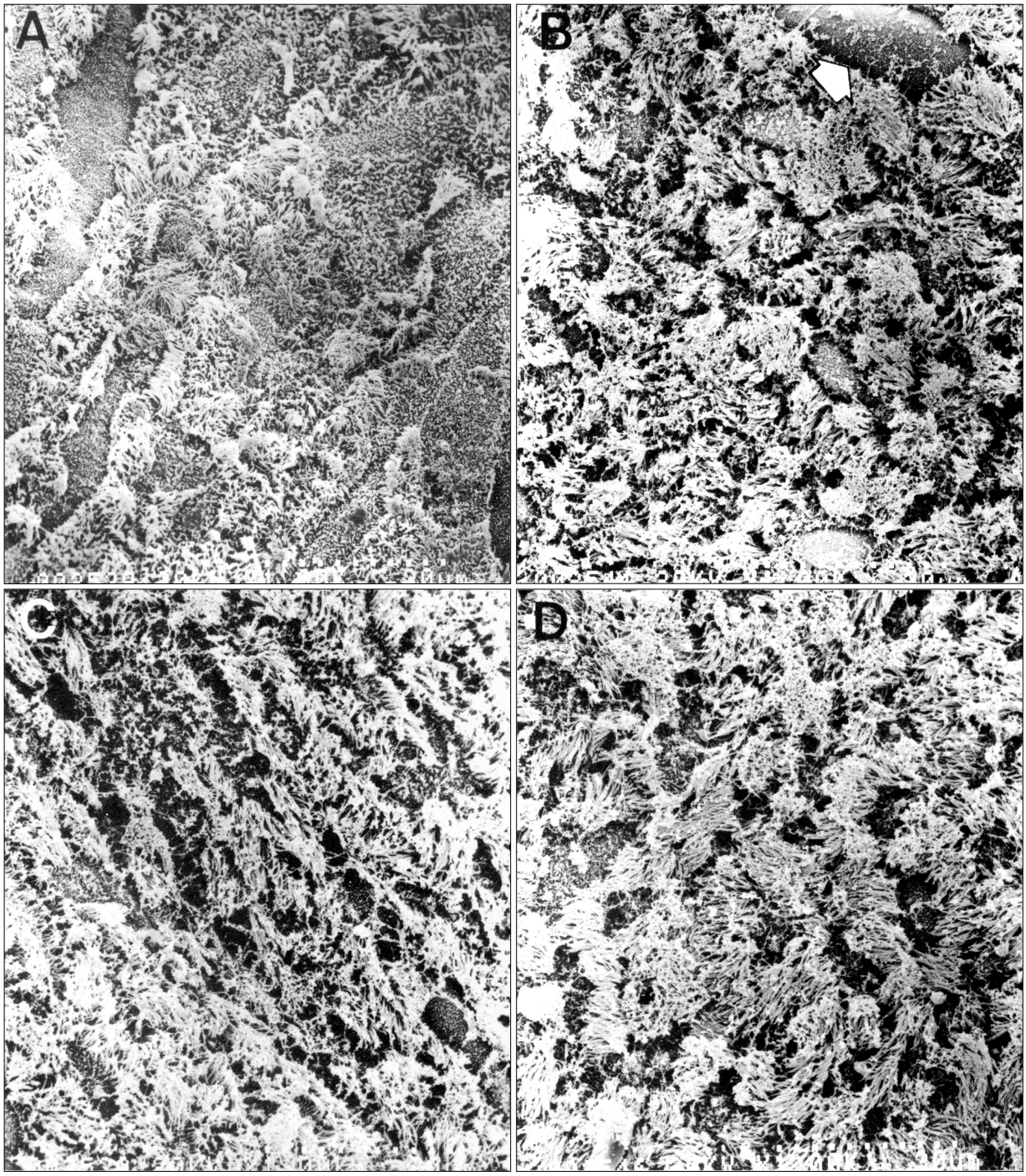

Fig. 1. Scanning electron microscopic findings according to the time sequence after cessation of adding 10 ng/ml TNF-a in the culture media for 48 hours. A. On the first day, the damaged cilia and increased secretory cell area are noted. B. On the third day, the recovery of cilia is observed, but the increased secretory cell area is still not normalized. C. On the fifth day, the cilia showes the normal appearance and the secretory cell area is normalized. D. On the seventh day, the cilia and the secretory cell areas appear as normal.

the culture media, which contained Dulbeco's modified Eagle medium and Ham's nutrient F 12 as the same ratio. Supplements - Cholera toxin (10 ng/ml : Sigma Chemical Co.), retinoic acid (10^{-7} Mol : Sigma Chemical Co.) and 10% NU serum (Collaborative Research Inc.) - were used.

Application of TNF-α and the observation of the reversibility

Recombinant human TNF-α (Gengyme Diagnostics, Cambridge, MA, USA) was used. The concentrations of TNF-α were 1, 10, 100 ng/ml and the cells without any added TNF-α was used as the control. We conducted the experiment using TNF-α at the concentration of 1, 10 and 100 ng/ml because those concentrations induced morphologic changes of HNEC according to our previous study.[8] The epithelial cells which were floated on the floating 14th day were selected for the experiment because they exhibited similar patterns of differentiation with those in the *in vivo* state.

For inducing the ultrastructural changes by TNF-α, epithelial cells were cultured for 48 hours in the media containing TNF-α as described in our previous study.[8] For observation of the reversibility according to the time sequences, the epithelial cells cultured in the media containing 10 ng/ml TNF-α for 48 hours were recultured in the normal media and observed on the first, third, fifth and seventh day. For observing the reversibility according to the concentration, epithelial cells at each concentration were cultured for seven days in the normal media.

The observations were carried out using scanning & transmission electron microscopes. For quantitating the areas of ciliated and secretory epithelial cells, ten scanning electron microscopic photos of 1000 magnification were taken and the areas per 60 μm^2 were calculated and statistically analyzed

Table 1. Area of ciliated and secretory epithelial cells according to the time sequence of culturing in normal media after culturing with TNF-α 10 ng/ml for 48 hours

	Area of ciliated cell (μm^2)	Area of secretory cells (μm^2)
Zero day	41.0 ± 5.6	19.0 ± 6.1
First day	40.7 ± 6.8	19.3 ± 6.0
Third day	42.1 ± 7.3	17.9 ± 6.2
Fifth day	47.7 ± 7.6*	12.3 ± 7.0*
Seventh day	48.7 ± 8.5*	11.3 ± 7.3*

* $p<0.05$ compared with zero day

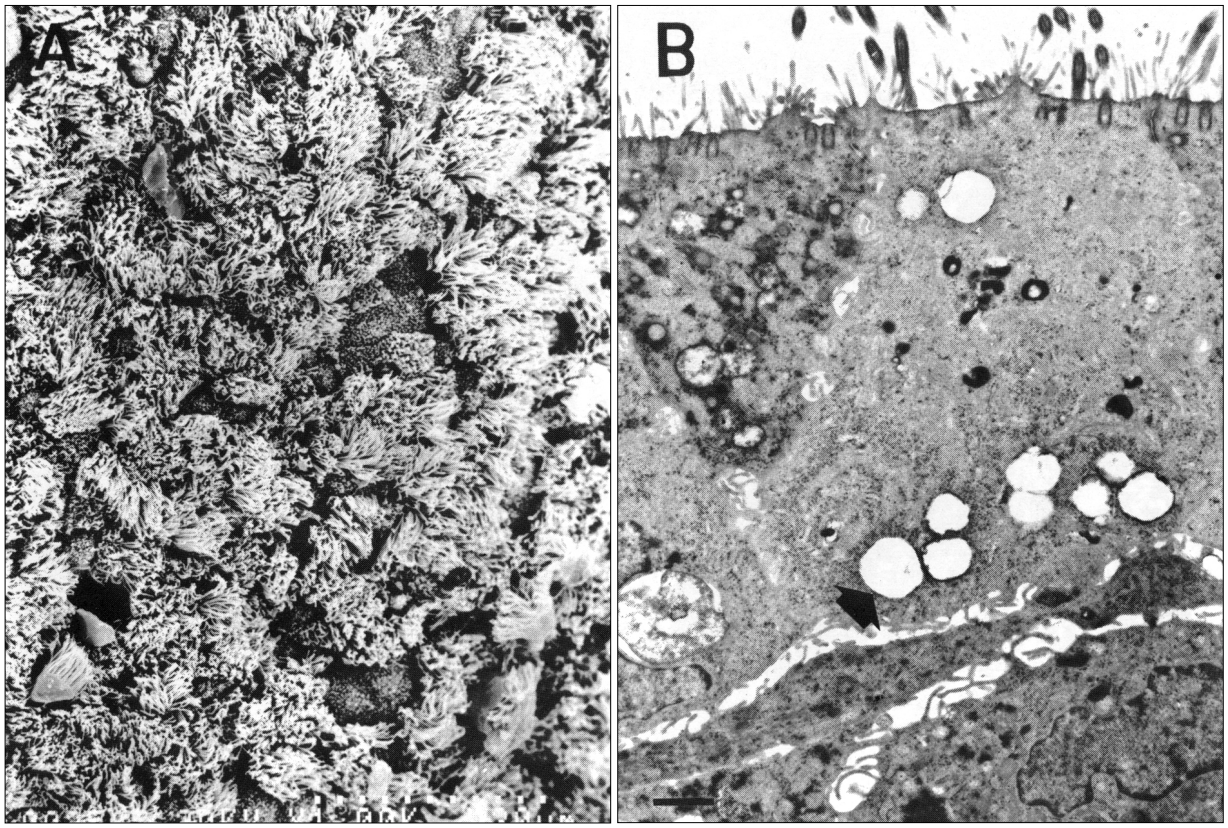

Fig. 2. Electron microscopic findings of normal control on the floating 23 rd day. A. Scanning electron microscopic finding : The distribution pattern of cilia is dense and the secretory cells are found in the multiple small areas bearing no cilia (×1000). B. Transmission electron microscopic finding : Many cilia are protruded from the cell surface and relatively tight intercellular spaces are shown. The intracellular granules of secretory cells are electron-lucent (arrow) (×7500, scale bar : 1 μm).

using SPSS/PC program (ANOVA).

RESULTS

Reversibility according to the time sequence

We conducted the experiment using TNF-α at the concentration of 10 ng/ml because that concentration induced the most typical morphologic changes of HNEC according to our previous study.[8] On 48 hours after the cessation of adding 10 ng/ml, morphologic changes such as damage of cilia and an increase of secretory cell area were noted with scanning electron microscopy. In transmission electron microscopy, damage of cilia, increase of mitochondria and intercellular space or intercellular vacuoles were observed.[8]

On the first day after cessation of adding 10 ng/ml TNF-α to the culture media, changes such as damaged cilia and increased secretory cell area were noted, which was similar to the changes observed on the zero day (Table 1)(Fig. 1A). On the third day, the recovery of cilia was observed, but the increased secretory cell area was not reduced (Table 1)(Fig. 1B). Concerning the surface area of cells, the secretory cell area was normalized on the fifth day (Table 1)(Fig. 1C) and maintained normal until the seventh day (Table 1)(Fig. 1D).

Reversibility according to the concentration

According to our previous study, the ultrastructural changes by TNF-α in HNEC were observed from 1 ng/ml through 100 ng/ml. Damage of cilia, an increase of mitochondria and either intercellular space or intercellular vacuoles were noted in transmission electron microscopy. As to the surface area of cells, the area of secretory cells was increased from 1 ng/ml through 100 ng/ml in scanning electron microscopy.[8]

On the seventh day after cessation of adding TNF-α to the culture media, the above changes in the epithelial cells were mostly reversed with the cells returning to normal status (Fig. 2) at the concentrations of 1 and 10 ng/ml (Table 2)(Fig. 3). However, the area of secretory epithelial cell was still larger

Table 2. Area of ciliated and secretory epithelial cells according to the concentration on the seventh day of culturing in normal media after culturing with TNF-a for 48 hours

TNF-a (ng/ml)	Area of ciliated cell (μm^2)	Area of secretory cells (μm^2)
TNF-a 0	48.4 ± 8.6	11.6 ± 6.3
TNF-a 1	47.9 ± 8.0	12.1 ± 6.6
TNF-a 10	48.3 ± 7.7	11.7 ± 6.7
TNF-a 100	39.8 ± 6.7*	20.2 ± 5.3*

* $p<0.05$ compared with TNF-a 0 ng/ml

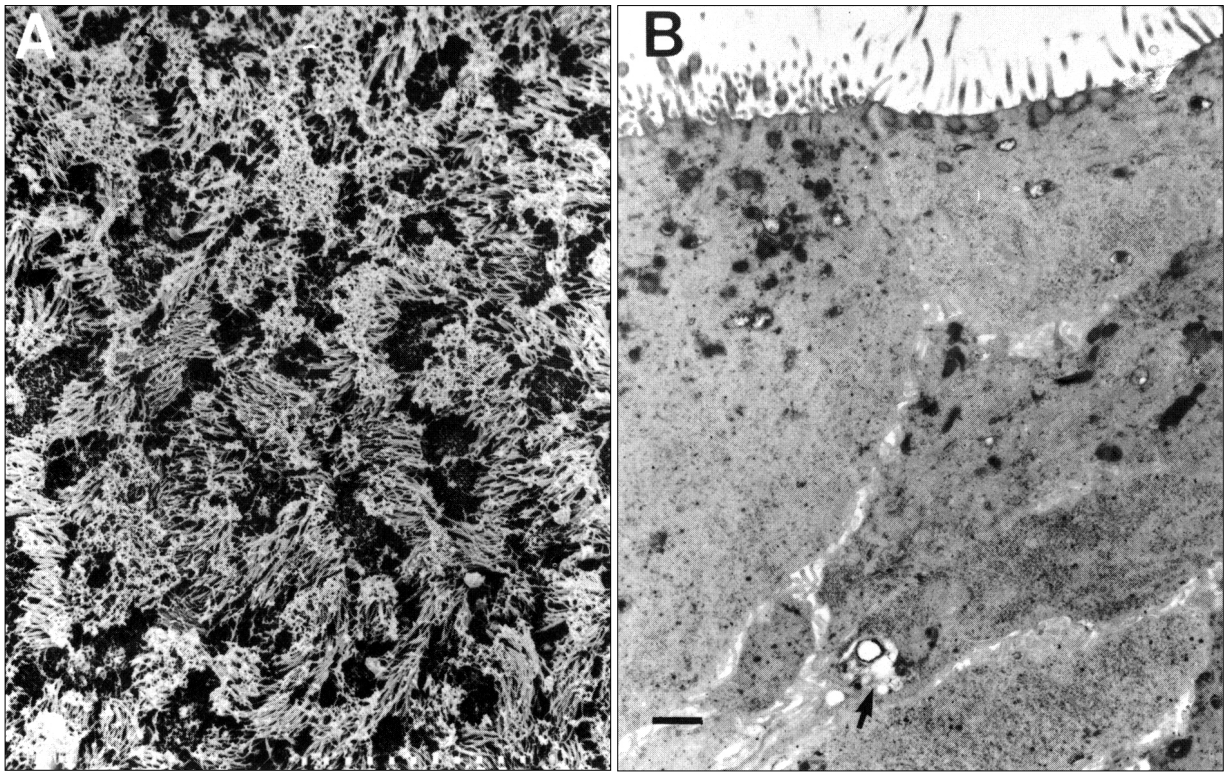

Fig. 3. Electron microscopic findings on the seventh day after the cessation of adding 10 ng/ml TNF-α. A. Scanning electron microscopic finding : The changes of epithelial cells return to normal (×1000). B. Transmission electron microscopic finding : Normal appearance without such changes as damaged cilia, increased number of mitochondria, intercellular spaces or intercellular vacuoles. The electron-lucent (arrow) intercellular granules are found (×5400, scale bar : 1 μm).

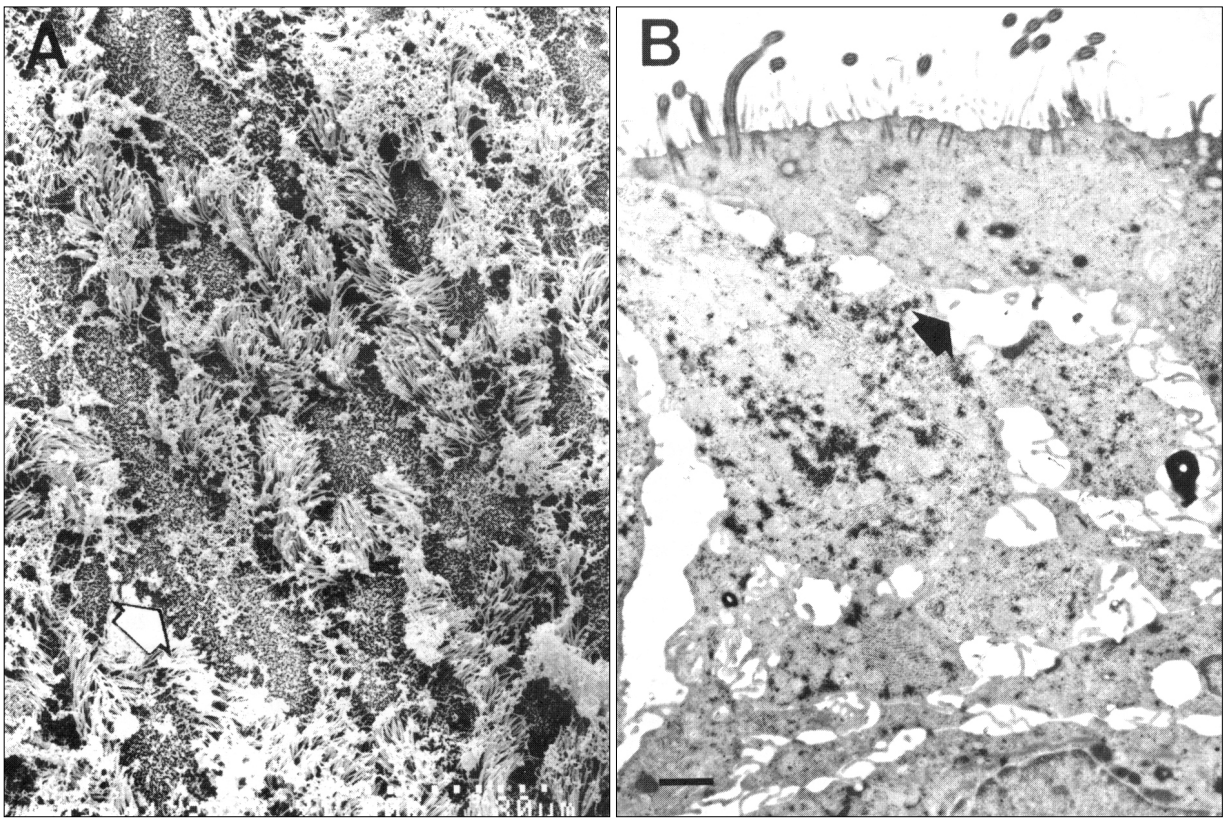

Fig. 4. Electron microscopic findings on the seventh day after the cessation of adding 100 ng/ml TNF-α. A. Scanning electron microscopic finding : The distribution of cilia is dense and appears to be normal, but the area of secretory epithelial cells is larger than control (arrow) (×1000). B. Transmission electron microscopic finding : Numerous numbers of cilia are seen and intercellular spaces, especially in the lower portion, are widened (arrow) (×7100, scale bar : 1 μm).

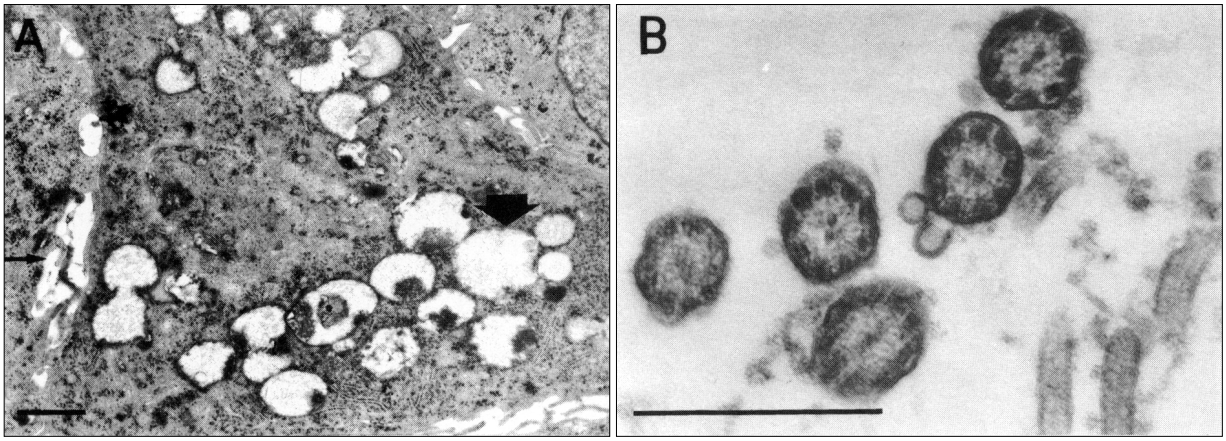

Fig. 5. Transmission electron microscopic findings on the seventh day after the cessation of adding 100 ng/ml TNF-α. A. The intercellular spaces are widened (thin arrow) and the intracellular granules are electron-lucent (thick arrow) (×12000, scale bar : 1 μm). B. The cilia show typical 9+2 patterns (×85000, scale bar : 1 μm).

than control at the concentration of 100 ng/ml TNF-α (Table 2) (Fig. 4). The secretory granules on the seventh day appeared mostly electron-lucent and the sectioned surface of cilia showed typical 9+2 patterns (Fig. 5).

DISCUSSION

Study of the pathophysiologic effects of TNF-α has been

intensively done but little information about the morphologic effects of TNF-α has been available until now. The morphologic effects of TNF-α on the epithelial cells have shown that TNF-α induces the widening of the tight junction of human renal epithelium and leads to the increase of transport through the membrane.[7] The activation of TNF-α was also reported to result in morphologic changes of the rat mammary gland epithelium[12] and bovine endothelial cells.[6] The previous work of authors confirmed that the morphologic changes of HNEC induced by TNF-α showed similar changes with the *in vivo* changes : damage of cilia, an increase of mitochondria and either intercellular space or intercellular vacuoles. Especially with the concentration of TNF-α 1 ng/ml, the ultrastructural changes noted were similar to the necrosis patterns among the inflammatory reactions *in vivo*.[8]

The first objective of this study was to observe the time of recovery from the morphologic changes induced by TNF-α. According to another study[6] on the time sequence of reversibility, the bovine endothelial cells recovered to normal shape on the third day after the cessation of adding TNF-α 1000 U/ml. In our study, the recovery was possible on the third day in the cilia and on the fifth day in the secretory cells. This results coincided with the study on the bovine endothelial cells with respect to the reversibility, but there was a discrepancy in time gap between two works, which may be the result of using the different experiment materials.

The second objective was to observe whether the changes are completely recovered. The changes induced by TNF-α 1 and 10 ng/ml were completely recovered on the seventh day. These facts are similar to the *in vivo* state, in which the tissues and cells were recovered to the normal morphology after inflammation.[9] Even though the ciliated cells were reversible, the secretory cell area was still larger than the control at the concentration of TNF-α 100 ng/ml. This incomplete recovery is likely due to the fact that our study was carried out only up to the seventh day. Thus, it is possible that the enlarged area of secretory cells may be reduced to normal size if observation is done for more than seven days. Study on the recovery after the seventh day will be needed to confirm this.

Although the concentration of TNF-α in the tissue of the patient with allergic rhinitis or infection is unclear,[13] the concentration of 100 ng/ml of TNF-α is very high considering the fact that the concentration of 109 ng/L was detected in the serum of a patient with septic shock.[14] Therefore, the reversible change or recovery of cilia is very interesting even though the origin of this reversibility is still doubtful. Recovery of rabbit septal mucosa after mechanical trauma occurred on the fifth day in the case of intact basal cells, but it occurred on the third week in the case of those with damaged basal cells.[15][16] In our study, the cilia were damaged, but the basal cells and the ciliated cells were morphologically intact. Furthermore, the appearance of electron-lucent secretory granules (Fig. 5) indicates that the biological activity of the cells likely remains nearly normal. According to the above report using the rabbit, the seven days over which our experiment was carried out is longer than necessary for the basal cells to differentiate into the mature epithelial cells, as long as the basal cells remain undamaged. Otherwise, it is possible that the basal cells may not be adequate to the task of helping the epithelial cells to recover from the damage. But although the ciliated epithelial cell is the final stage of differentiation *in vivo*, it can be differentiated into other types of cells such as secretory cells or basal cells in the cell culture system. Furthermore, even the secretory cells, also the final stage cell *in vivo*, can be differentiated into ciliated cells[17] in the cell culture system.

Therefore, the source of cellular recovery can not be precisely defined at this time, and further studies would be required before any conclusions can be made regarding the significance of the role played by basal cells in the recovery of epithelial cells.

CONCLUSION

The authors were to observe how long it took nasal epithelial cells to recover from the changes induced by TNF-α and determine whether or not varying degrees of changes effect whether the cells completely return to normal. The recovery of cilia was observed on the third day after cessation of adding TNF-α and the recovery of secretory cell area on the fifth day. The morphologic changes induced by TNF-α were reversible except at the concentration of 100 ng/ml.

The results of this study suggest that the ultrastructural changes induced by TNF-α are mostly reversible and this reversibility corresponds with the *in vivo* state. Further study on the recovery after the seventh day at the concentration of TNF-α 100 ng/ml might be needed.

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